Research Article

Research on the Structure of Fish Collagen Nanofibers Influenced Cell Growth

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Electrospinning is highlighted in biomaterials field. The structures of nanofibers depend on various parameters, which are related closely to the bioactivity of biomaterials. The aim of this research is to analyze the structure of fish collagen nanofibers and to propose the new criterion for cell growth. This paper focused on the flow rate of solvent during the electrospinning. Through the cell culture, the relationship of the structure and cell growth is investigated. The results obtained in this study provide an understanding of the behaviors of cell growth under different structure of fish collagen nanofibers scaffold.

1. Introduction

Recently, electrospinning is gaining tremendous interest in the tissue engineering [1, 2]. Electrospinning has been explored as a processing strategy for the production of physiologically relevant tissue engineering scaffolds. This adaptable technology can selectively process a variety of polymers [3, 4] into nanosize to micron scale diameter fibers. These nanofibers can mimic the dimensions, structure, and function of native extracellular matrix (ECM) constituents [5]. Moreover, the electrospun nanofibers have large surface area to volume ratio, which attached ligands [6], growth factors [7], and other biomolecules [8] onto fiber surfaces. Then, these factors locally modulate cell and tissue function and lastly enhance tissue regeneration [9, 10].

For tissue engineering applications, collagen-based compounds are very suitable, because they possess some intrinsic favorable biological properties, like superior biocompatibility and bioactivity [11]. Collagen molecules have a triple-helical structure and the presence of 4-hydroxyproline resulting from a posttranslational modification of peptide-bound prolyl residues provides a distinctive marker of these molecules

[12]. Until now, 28 collagen types have been identified; I, II, III, and V are the main types that make up the essential part of collagen in bone, cartilage, tendon, skin, and muscle. They also exist in fibrillar forms with elaborate 3D arrays in ECM [13]. From the biomimetic point of view, collagen has been electrospun into nanofibrous scaffolds for tissue engineering [14, 15]. However, the main sources of collagen are usually from terrestrial animals, such as porcine and bovine donors. There are some zoonosis outbreaks [16–18], such as transmissible spongiform encephalopathy (TSE), bovine spongiform encephalopathy (BSE), and foot and mouth disease (FMD) in pigs and cattle. Thus, collagen derived from these sources has limited. Therefore, safer sources of collagen should be utilized for the biomaterials field. Compared with terrestrial animals, hydrocele animals are good alternative resources for collagen. Fish scale is composed of almost 50% collagen, mostly type I. It was highlighted due to its low antigenicity and excellent biocompatibility [19]. Consequently, in this study a novel fish collagen was investigated that finally can be used as an electrospun material.

The electrospun parameters not only affected the average diameter of nanofibers, but also the structure of scaffolds.

FIGURE 1: FT-IR spectrum of fish collagen.

Few papers researched on the cell growth under the different structure of nanofibers in scaffolds. In the present study, the electrospinning method was used to prepare fish collagen nanofibers. The properties of these nanofibers were characterized and analyzed by Fourier transform-infrared (FT-IR) and scanning electron microscope (SEM). Moreover, cell culture experiment was done to investigate the relationship between cell and different structure of this material.

2. Materials and Methods

2.1. Materials. *Tilapia mossambica* was bought from supermarket. It is healthy.

2.2. Preparation of the Fish Collagen. The fish scales were scraped from *Tilapia mossambica*. They were trimmed of fur and excess fat. Then, strips of scales were washed and cleaned by distilled water repeatedly 5 times, drying at 25°C.

The dry scales placed in distill water and treated for 1 min in a commercial flash extractor (Model JHBE-50T, Henan Jinnai Sci-Tech Development Co., China). The production was dissolved in 0.5 M acetic acid, stewing in room temperature for 4 h, and then centrifuged at 5000 r/min for 15 minutes. The liquid supernatant was harvested filtering; solid NaCl was added to the liquid supernatant to a final concentration of 0.9 M, incubated at 4°C for 12 h. Then, the solution was centrifugation at 5000 r/min for 20 min to remove all nonsoluble collagen fragments. Materials were separated by adding NaCl to a concentration of 0.9 M to induce precipitation. The precipitated collagen was collected by centrifugation at 5000 g for 20 min, redissolved in distill water, and dialyzed for 4 days. The final collagen solution was freeze-dried and stored at −20°C until further use.

2.3. Electrospinning of Fish Collagen. Fish collagen was dissolved in hexafluoro-2-propanol (HFIP; Fluka Chemie GmbH, Germany) to generate a 10% (wt/v) solution. Electrospinning was performed with a steel capillary tube with

a 2 mm inside diameter tip mounted on an adjustable, electrically insulated stand. The capillary tube was maintained at a high electric potential for electrospinning and was mounted in the parallel plate geometry. The capillary tube was connected to a syringe filled with the fish collagen/HFIP solution.

During this step, the ambient temperature was $25 \pm 1^\circ\text{C}$, the humidity was $58 \pm 2\%$. The distance between collection plate and needle tip was 15 cm. The applied voltage was 15 kV. Nanofibers were electrospun on the gap collector by adjusting the flow rate of fish collagen solution at different variants using a syringe pump. The flow rate of collagen solvent was 0.1 m/s (indicate I); 0.5 m/s (II); 1.0 m/s (III); 1.5 m/s (IV), respectively.

2.4. Characterization of Materials

2.4.1. FT-IR Analysis. FT-IR spectroscopic analysis of fish collagen was performed using a Thermo Nicolet 170SX FT-IR Spectrometer in the region from 400 to 4000 cm^{-1} (spectral resolution 4 cm^{-1} , 32 scans per spectrum).

2.4.2. SEM Observation. The surface morphology of fish collagen scaffold was viewed under a scanning electron microscope (Hitachi S-450), with an accelerated voltage of 10 kV. All samples were coated with a sputter coater (BAL-TEC Inc.) equipped with a gold target to increase electrical conductivity. The average diameter and diameter distribution of fish collagen nanofibers were measured from SEM images by ImageJ program, with an accuracy of $\pm 0.01\text{ nm}$. All the samples have been measured using the 5 different SEM images.

2.4.3. Cell Culture. HEK-293 cells were cultured and maintained in logarithmic growth phase in DMEM (Gibco, USA) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO_2 . Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-[2H]-tetrazolium bromide (MTT) assay. The dye MTT is taken and metabolized to purple by viable mitochondria. Cells were counted and plated in 96-well plates at a rate of 1×10^4 cells per well and incubated in 200 μL cell culture medium. Prior to dilution with culture medium, the fish collagen was sterilized by UV light for 12 h. After incubation for 1, 3, 5, and 7 days, 20 μL MTT solution (5 mg/mL, prepared with PBS, pH 7.4) was added to each well and incubated at 37°C and 5% CO_2 for an additional 4 h. The purple MTT was dissolved in 150 μL dimethyl sulfoxide solution (DMSO) (Sigma, USA). The activity of the mitochondria, reflecting cellular viability, was evaluated by measuring the optical density at 490 nm using an ELISA microplate reader (Bio-Rad Instruments, Inc.). The cell viability (%) of the treated cells was calculated in relation to the negative controls (100%).

2.5. Statistical Analysis. Data are given as means $\pm$ S.E.M. For statistical comparison, *t*-test or one-way ANOVA followed by Tukey's test was employed. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ were considered to be statistically significant.

FIGURE 2: SEM images of electrospun fish collagen when the flow rate of solvent was (a) $v = 0.1$ mL/h (indicate I); (b) $v = 0.5$ mL/h (II); (c) $v = 1.0$ mL/h (III); (d) $v = 1.5$ mL/h (IV).

3. Result and Discussion

3.1. FT-IR Analysis. The FT-IR spectrum of collagen depicts characteristic absorption bands at 1634 , 1544 , and 1250 cm^{-1} , which represent the amide I, II, and III bands of collagen [20]. The main amide III characteristic absorption band is observed at 1250 cm^{-1} , with another peak seen at 1335 cm^{-1} , as shown in Figure 1. The result of FT-IR measurement indicates the isolation of fish collagen that can be accounted for Type I collagen.

3.2. SEM Observation. Nanofibers were manufactured from fish collagen solution of 4% (w/v) concentration. The first series of experiments were carried out at the flow rate of solvent 0.1 mL/h, the second 0.5 mL/h, the third 1.5 mL/h, and the fourth 1.5 mL/h. The morphologies of scaffold were presented in Figure 2. Each variant produced different morphologies of fibers (shown in Figure 2). With the flow rate increase, system produced a population that was skewed towards the production of larger diameters during the electrospinning.

During the observation, the four SEM images for each variant of flow rate were detected, respectively. The 146 ± 57 diameters of fish collagen nanofibers for the I variant, 121 ± 17 diameters of nanofibers for II variant, 283 ± 12 diameters of nanofibers for III variant, and 318 ± 30 nanofibers for IV variant were found and measured. The structure of fish collagen scaffold also depends on the flow rate. The electrospun fish collagen nanofibers were parallel aligned without defects at II variant (0.5 mL/h). At the 0.1 mL/h, the nanofibers were random. At the flow rate of 1.0 mL/h, the surface of nanofiber was rather roughness (shown in Figure 2(c)). It resulted from some nanofibers get together. At the highest flow rate (1.5 mL/h) of solvent, small diameter fibers interspersed with beads and stuck together, composing the larger diameter fibers and highly aligned. This change would decrease the porosity of scaffold.

In the next step of investigations, the dispersions of fish collagen nanofibers have been studied. The frequency distributions of all series were discussed in Figure 3. Four histograms demonstrated that the diameter of nanofibers

in all cases was distributed in different distributions. In Figure 3(a), three peaks were remarked observed: the first peak was around 50 nm, the second peak was around 100 nm, while the third was 150 nm. A similar result, the distribution with several peaks, could be detected in Figure 3(b): the first peak is around 75 nm, the second is around 100 nm, and the third is 125 nm. With the flow rate increase, the histogram really has one main peak around 300 nm. These changes were the sticking of fish collagen nanofibers. During the electrospinning process, the nanofibers were manufactured by means of electrostatic forces between two electrodes [21]. The diameters of nanofibers usually changed and they depend on various parameters. The different diameters of fibers influenced the structure of scaffold and herewith the end-use properties of such kind of nanomaterial. Therefore, the flow rate of solvent was a very important parameter for the structure of nanofibers. Using the higher flow rate, more stuck fiber scaffolds were forming.

3.3. MTT Evaluation. Cell proliferation on the collagen was studied in vitro by MTT test. Because of the differences in the chemical composition, conformation, porosity, and hydrophobicity of matrix affect cellular activities [22, 23], time-dependent changes were observed in the cellular behavior in response to collagen compositions. In 7-day cell cultures, the cell number increased with culture time on all tested groups (Figure 4). While at day 1 there was no statistically significant difference among the collagen and the control groups, at the later time point (5th day for HEK-293 cells), the cell numbers on collagen were significantly higher than those cocultured with control, which implied that the fish collagen favored cell attachment. Fish collagen nanofiber scaffolds exhibited excellent support for cell attachment and differentiation. In addition, there was a trend showing the highest number of cells in II variant group compared to others. Electrospun scaffolds of collagen composed of small diameter fibers induced the expression of a highly flattened and stellate cell shape at this flow rate. Critical to the bioengineering paradigm is the development of tissue engineering scaffolds that can support the proliferation and penetration [24]. While at the highest flow rate of solvent cell

FIGURE 3: Distributions of electrospun fish collagen during the experiment was (a) $\nu = 0.1$ mL/h (indicate I); (b) $\nu = 0.5$ mL/h (II); (c) $\nu = 1.0$ mL/h (III); (d) $\nu = 1.5$ mL/h (IV).

grew least in the collagen groups. It means that the different structure fiber scaffolds significantly affect cell growth. At higher flow rate, the fiber stuck together, resulting in porosity decreased. There is too little room to help cell attachment and growth in the scaffold.

Scaffold design [25–33] is to produce an ideal structure that acts as an ECM until host cells can repopulate and resynthesize a new natural matrix. Collagen, as the basic structural element for most connective tissues, plays a prominent role in maintaining the biologic and structural integrity of ECM architecture and presents different morphologies in different tissues which perform different functions. Particularly, fish collagen biomaterials formed instructive extracellular microenvironments for morphogenesis in tissue engineering. Particularly fish collagen is preferred for cell attachment and proliferation, which would be widely useful for tissue regeneration. Further application researches will focus on them.

4. Conclusions

In this study, the fish collagen was firstly isolated by flash extraction. The Type I collagen was characterized by FT-IR. It was also found as an appropriate flow rate of solvent for electrospinning. This parameter depended on the diameter and structure of nanofibers scaffold. Further, it significantly affected cell growth.

Conflict of Interests

The authors declare no possible conflict of interests.

Authors' Contribution

Gang Zhou and Guimin Zhang equally contributed to this paper.

FIGURE 4: MTT assays for proliferation of HEK-293 cells co-cultured with fish collagen at 1, 3, 5, 7 days (Control indicate co-cultured without materials; I indicate $\nu = 0.1$ mL/h; II indicate $\nu = 0.5$ mL/h; III indicate $\nu = 1.0$ mL/h; IV indicate $\nu = 1.5$ mL/h).

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